# Molecular subtyping of *Salmonella* Typhimurium combining different types of markers in a multiplex liquid bead suspension array

*Véronique WUYTS[1,2], Sophie BERTRAND [3], Nancy ROOSENS [2], Kathleen MARCHAL [1,4] and Sigrid DE KEERSMAECKER [2]*
*[1]Department of Microbial and Molecular Systems, KU Leuven, Leuven, Belgium;*
*[2]Platform Biotechnology and Molecular Biology, Scientific Institute of Public Health (WIV-ISP), BRUSSELS, Belgium;*
*[3]National Reference Centre for Salmonella and Shigella, Bacterial Diseases Division, Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), BRUSSELS, Belgium;*
*[4]Department of Plant Biotechnology and Bioinformatics, Ghent University (VIB), GENT, Belgium*

## ABSTRACT
Subtyping of *Salmonella enterica* subsp. *enterica* serovar Typhimurium, a frequent cause of food-borne diseases, is critical for surveillance and identification, tracking and ultimately confinement of outbreaks. We present a preliminary proof of concept of a molecular subtyping method for *S.* Typhimurium which combines different types of markers in a multiplex assay with detection on a liquid bead suspension array in a high-throughput format. Selected markers include, amongst others, markers based on AFLP fragments, prophage genomes, sequence repeats, antibiotic resistance genes and SNPs. The optimal multiplex design was evaluated and a ligation dependent amplification (LDA) assay was found to be the most efficient strategy to target the selected markers. The *in vitro* stability of the selected markers was verified. To determine the discriminatory ability, *S.* Typhimurium isolates of most common phage types in Belgium were subjected to the proposed subtyping method. The capability to identify outbreaks was tested with strains from two outbreaks in Belgium that occurred in 2008 and 2011. The resulting profiles were examined for correlation with phage types, MLVA and antimicrobial resistance profiles. The potential of the new method to provide a new subtyping scheme for *S.* Typhimurium will be discussed.

## INTRODUCTION
Classically in surveillance, isolates of *Salmonella enterica* subsp. *enterica* serovar Typhimurium are phage typed and, often in outbreak situations, further subtyped by molecular techniques as pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem repeats analysis (MLVA) or multilocus sequence typing (MLST). Although proven to have additional value for subtyping, each of these techniques has its intrinsic disadvantages and scientists still search for an ideal subtyping method, which should be inexpensive, rapid, highly discriminative and robust (2, 4).
Until now, different kinds of DNA markers have been studied, alone or combined, for their suitability for a molecular subtyping scheme for *S.* Typhimurium. The number of markers included in the resulting methods and the level of multiplexing still feasible, often determines the discriminatory power and rapidity (4).
In this study we deliver a preliminary proof of concept for a molecular subtyping method for *S.* Typhimurium that combines different types of markers, such as amplified fragment length polymorphisms (AFLP), prophage genomes, sequence repeats, antibiotic resistance markers and SNPs. To achieve a sufficient level of multiplexing in a high-throughput manner, we make use of the Luminex technology. This technology, which emerged in the 1990's, allows distinguishing up to 500 different targets in one sample through its bead-based assays.

## MATERIALS AND METHODS
### Bacterial isolates
*S.* Typhimurium isolates were selected from the collection of the Belgian National Reference Centre for *Salmonella* and *Shigella*. The selection includes 100 isolates, of which 13 isolates from two outbreaks, with different combinations of phage type, antimicrobial resistance pattern and MLVA profile. Besides the most frequent phage types over the past years in Belgium, *i.e.* DT12, DT104, DT120, DT193, DT195 and U302, also phage types DT1, DT35, DT138 and one reactions-do-not-

conform (RDNC) isolate are represented. In total, these isolates cover 26 different antimicrobial resistance patterns and 55 distinct MLVA profiles.

**DNA isolation**

DNA was isolated by dissolving a single colony from an overnight culture on LB agar into 300 µl sterile water and incubating at 100°C for 10 minutes. After cooling to 4°C and centrifugation for 10 minutes at 10,000 rpm, the supernatant was stored at -20°C and used for further analysis.

**Stability experiment**

Stability of selected markers was evaluated in 31 *S.* Typhimurium isolates of the most frequent phage types in Belgium. A series of 50 passages, starting from a single colony, was performed. Glycerol stocks were made before each fifth passage and DNA was isolated from cultures after the final passage (3).

**Marker selection**

Markers were taken from literature. Markers that are informative through presence or absence were screened by PCR and gel electrophoresis on 30 isolates of the most common phage types in Belgium. Those markers which were not present or absent in all 30 isolates and thus have discriminatory power, were selected for the liquid bead suspension array development.

**Bead-based nucleic acid assay selection**

The Luminex technology, which is implemented through a MAGPIX platform in this study, utilizes polystyrene beads with a different colour code for each set. Each bead set can be coated with a different capture probe. Multiplexing is then achieved by combining different bead sets to analyze one sample. During analysis, the set to which the bead belongs is determined by measuring the red fluorescence signal from the bead. Afterwards, the presence of a hybridized target oligonucleotide is detected by measuring the green fluorescence signal on that target. Each bead-based assay should thus incorporate a fluorophore in the target oligonucleotide. Hereto, different possibilities exist. Three bead-based assay formats were compared with regard to the usage possibilities, multiplexing capacity, workflow, optimization and cost.

The direct hybridization assay starts with a multiplex PCR with labelled primers, after which the products are hybridized to beads, to which capture probes were coupled. If primers labelled with biotin are used, an incubation step with streptavidin-phycoerythrin (SAPE) is necessary before analysis on a Luminex platform.

In contrast to the xMAP® technology that is used in direct hybridization assays where self-designed capture probes have to be attached to the beads, the xTAG® technology utilizes beads with pre-coupled 24 bp anti-TAG-sequences. Allele specific primer extension (ASPE) and ligation dependent amplification (LDA) are built upon this technology and should integrate the complementary TAG-sequence in the target oligonucleotide.

ASPE commences with a multiplex PCR, after which the products are treated with ExoSAP-IT to degrade remaining primers and dNTPs. Target specific probes with a TAG-sequence at 5' are then annealed to the PCR product and extended with inclusion of biotin-dCTP. The ASPE products are analyzed on a Luminex platform after hybridization to xTAG beads and incubation with SAPE.

The first step in a LDA assay (figure 1) is a multiplex ligation with a probe pair for each target. The upstream probe has a universal primer site, TAG-sequence and target specific part, the adjacent downstream probe has a target specific part and universal primer site. The second step comprises a singleplex PCR with universal primers, one of which is labelled. After hybridization to xTAG beads and an optional incubation with SAPE, the amplified ligation products are read out on a Luminex platform.

**Multiplex oligonucleotide design**

Oligonucleotides for the multiplex liquid bead suspension array were designed with the Visual OMP software (DNA Software).

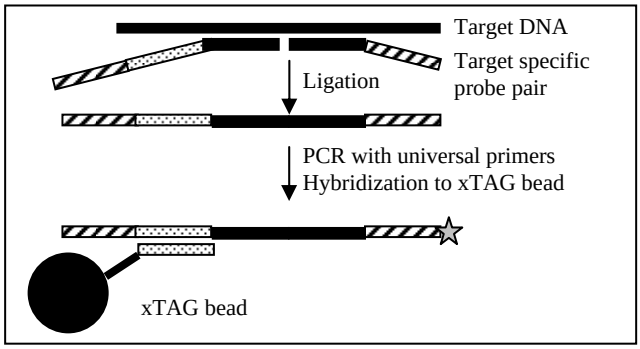


*Figure 1: Ligation dependent amplification (LDA). Probes have a target specific part (black), a universal primer site (striped) and a TAG-sequence (dotted). A fluorescent label (star) is introduced via a labelled universal primer.*

**RESULTS**

**Marker selection**

Through PCR screening on 30 isolates of phage types DT12, DT104, DT120, DT193, DT195 and U302, 34 literature-based markers, detecting AFLP fragments, prophage genomes, sequence repeats, SGI1 and allantoin utilization, have been found informative.

Also included in the current selection of markers, but not screened through PCR, are antibiotic resistance genes, SNPs and a marker specific to *Salmonella enterica* subsp. *enterica*.

**Bead-based nucleic acid assay selection**

To combine the selected markers in a multiplex liquid bead suspension array, three nucleic acid assay formats were considered. Advantages and disadvantages of the different bead-based nucleic acid assays are summarized in table 1. The LDA assay was chosen for development of a subtyping method for *S.* Typhimurium, since the multiplex step in this assay format is a ligation, which allows a higher multiplex capacity and an addition of markers without redesign of the assay, in contrast to multiplex PCR. Additionally, the LDA does not require the use of neurotoxic TMAC buffer, of coupling of capture probes and of optimization of the hybridization to Luminex beads. Also the costs and total time to complete the assay were found to be acceptable.

**Preliminary proof of concept**

From the 34 markers found informative through PCR screening, 5 markers with high discriminatory power, including prophages and SGI1, were selected for the first LDA tests. A marker detecting *Salmonella enterica* was included as a control for the DNA templates.

The resulting 6-plex LDA was applied to 100 isolates and the 31 isolates after 50 passages in the stability experiment. The amplified ligation products were read out on a MAGPIX platform. Each assay included a negative control, containing all reagents but no DNA template, for background signal measurement, and a positive control, containing all reagents and a mixture of DNA template representing all 6 markers, to verify the reaction. A clear separation was observed between negative and positive signals, measured as mean fluorescence intensity (MFI), which is illustrated by the boxplots in figure 2.

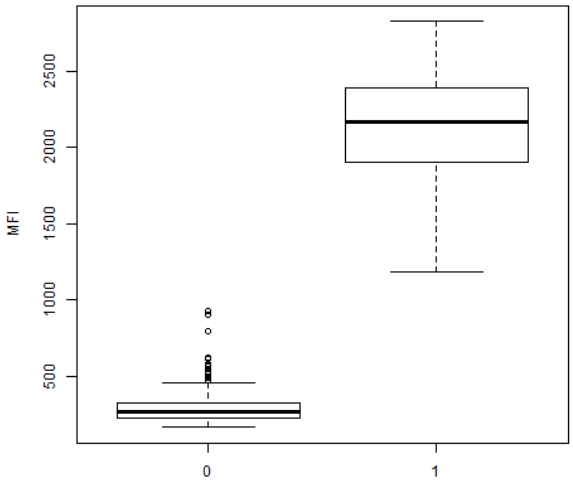


*Figure 2: Boxplots of MFIs of negative (0) and positive (1) signals of amplified LDA products analyzed on a MAGPIX device*

Presence or absence of markers, as detected by the LDA, resulted in 7 distinct profiles in the 131 tested isolates. The PCR profiles of the 30 isolates used in the PCR screening of markers, all correspond with the profile determined by the LDA. The isolates in the stability experiment gave an identical profile before and after the 50 serial passages in LB medium, thereby demonstrating the stability of the 6 markers included in the assay.
All DT104 isolates had the same profile, differing from the one DT104A isolate tested. Some isolates of DT12 and U302 shared the same profile as the DT104 isolates, others were found with different profiles. Isolates of DT193 with 2, 3 or 4 repeats at MLVA locus STTR9 showed distinct LDA profiles; the profile of the isolate with 4 repeats was not shared with other isolates.
With the current 6 selected markers, correlation with antimicrobial resistance patterns could not be observed and also no distinct profile was obtained for the outbreak isolates. These findings necessitate extending the LDA assay with more informative markers to a higher level of multiplexing.

**DISCUSSION**

Many of the proposed molecular methods for subtyping of *S.* Typhimurium are limited by the number of molecular markers and by the capacity of multiplexing those markers in a single run. The LDA assay can achieve a high level of multiplexing by separating detection and amplification. LDA is also cost-effective since the specific probe pairs are unlabelled. Numerous types of molecular markers, including SNPs, can be combined and straightforwardly added or left out of the assay. New probe pairs are assigned a different TAG for detection on the Luminex platform, but still exploit the universal primer pair for amplification of the signal, which makes the LDA a modular assay. In addition, LDA is very specific due to severe constraints on the ligation reaction: the upstream probe must anneal adjacent to the downstream probe and a strict complementarity is necessary for the base pairs flanking the ligation site. Amplification bias, as might be observed in multiplex PCR, is unlikely to affect LDA, since ligated probe pairs have about the same length (1).
Processing of the results generated by the LDA assay is facilitated by the single file output of the Luminex platform, which can easily be handled without the need of expensive software.

**CONCLUSION**

We have presented a preliminary proof of concept for the use of a LDA assay with analysis on a MAGPIX platform for the subtyping of *S.* Typhimurium. More markers will be included to increase the

discriminatory power and optimization of the workflow and of the assay will further reduce cost and time. This should lead to a cost-effective, rapid, highly discriminative, known target-based, and robust method, more efficiently addressing the needs of *S.* Typhimurium subtyping than MLVA and phage typing.

**ACKNOWLEDGEMENTS**

We would like to thank G. De Laminne de Bex for executing the stability experiment, C. Wildemauwe for phage typing, and the technicians of the Belgian NRC for *Salmonella* and *Shigella* for antimicrobial susceptibility and MLVA testing.

**REFERENCES**

1. Deshpande *et al.* (2010). J. Microbiol Methods, 80: 155-163.
2. Sabat *et al.* (2013). Euro Surveill, 18: pii=20380.
3. Struelens *et al.* (1996). Clin Microbiol Infect, 2: 21-31.
4. Wattiau *et al.* (2011). Appl Environ Microbiol, 77: 7877-7885.

*Table 1: Overview of advantages (+) and disadvantages (-) of direct hybridization, ASPE and LDA assays. mPCR denotes multiplex PCR.*

| Feature | Direct hybridization | ASPE | LDA |
|---|---|---|---|
| Usage | Unrelated sequences, SNPs, multiple polymorphisms | | |
| Multiplex step | - mPCR | - mPCR | + Ligation |
| Addition of marker | - Redesign | - Redesign | + No redesign |
| Buffers | - TMAC | + Tm | + Tm |
| Coupling of capture probe | - Yes | + No | + No |
| Hybridization to beads | - Optimize: 45-55°C | + Standard: 37°C | + Standard: 37°C |
| PCR amplicon | - < 300 bp | + all sizes | + all sizes |
| Patent | + No | + No | - Yes |
| Cost | ± | ++ | ± |
| Total time | + 3.5 hours | ± 7 hours | ± 6 hours |